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Three-Dimensional Ultrasound Versus Computerized Tomography in Fat Graft Volumetric Analysis

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Abstract

Studies evaluating fat grafting in mice have frequently employed micro-CT as an accurate radiographic tool to measure longitudinal volume retention without sacrificing the animal. Over the past decade, however, micro-ultrasonography has emerged as an equally powerful preclinical imaging tool. Given their respective strengths in 3D reconstruction, there is no study to our knowledge that directly compares micro-CT to micro-ultrasound in volumetric analysis. In this study, we compared the performance of micro-CT to micro-ultrasound in the evaluation of adipose tissue graft volume in a murine model. Fifteen immunodeficient mice were given 200 μ L adipose tissue grafts. *In vivo* volumetric analysis of the grafts by micro-CT and micro-ultrasound was conducted at discrete time points up to postoperative day 105. Three mice were sacrificed at multiple time points and explanted grafts were re-imaged by CT and ultrasound, as above. Analysis revealed that *in vivo* graft volumes measured by micro-CT do not differ significantly from those of micro-ultrasound. Furthermore, both micro-CT and micro-ultrasound were capable of accurately measuring fat grafts as *in vivo* volumes closely correlated with explanted volumes. Finally, ultrasound was found to yield improved soft tissue contrast compared to micro-CT. Therefore, either modality may be employed depending on experimental needs.

Keywords

Computed Tomography; Ultrasound; Fat Grafting; Volume; Soft-tissue Reconstruction

Introduction

The use of animal models has revolutionized the study of human disease. Among the myriad endeavors leveraged by comparative models, researchers have elucidated mechanisms of

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human diseases, created high-fidelity models of human physiology, and have streamlined pharmaceutical development and testing (1-5). The adaptation of sophisticated imaging modalities, once exclusive to humans, to the scale of small animals has transformed preclinical imaging into an integral, often irreplaceable component of modern comparative research. The current state-of-the-art in preclinical imaging encompasses modalities optimized for the study of anatomy, most notably bone and soft tissue. These technologies include micro-computerized tomography (micro-CT), micro-magnetic resonance imaging (micro-MRI) and micro-ultrasonography (micro-ultrasound) (1). As in human subjects, functional metabolic and molecular imaging in animals is accomplished with nuclear medicine studies, specifically positron emission tomography (PET) or single-photon emission computed tomography (SPECT), both alone as well as in tandem with existing tomographic techniques (i.e. PET/CT, PET/MRI, SPECT/CT) for concurrent visualization of anatomic structures (1). One of the most impactful contributions of preclinical imaging to comparative research has been the rapid advancement of non-invasive, non-destructive *in vivo* analysis. The aforementioned technologies have enabled longitudinal surveillance of subjects while reducing time and resource-consuming animal sacrifice and histology (2, 3).

The most ubiquitous preclinical imaging modality to date is micro-CT. X-ray tomography was pioneered by Hounsfield in the 1970's, and refined into the submillimeter resolution-capable machines of Kujoory *et al.* in 1980 and Flannery in 1987 (3, 6, 7). In micro-CT, hundreds of two-dimensional (2D) planar images are produced by passing X-rays from an emitter tube through the subject and onto a high-resolution detector. The X-ray tube emitter and detector are mounted on opposing sides of a circular gantry. This assembly is rotated in a 360-degree arc around a subject in a fixed position between the emitter and detector. The transmitted X-rays are attenuated variably depending on tissue density. Computer algorithms reconstruct the two-dimensional data set into a three-dimensional (3D) image, the smallest element of which is termed a voxel, the 3D correlate to the 2D pixel. Modern micro-CT scanners are capable of resolving voxels as small as 50 μm without contrast (1, 3), and even smaller voxels with contrast (8).

The birth of modern preclinical ultrasound imaging has been attributed to Sherar *et al.* and their work in characterizing living tumor spheroids (9, 10). Over the past decade, micro-ultrasonography – also referred to as micro-ultrasound – has emerged as a viable and powerful preclinical imaging tool complementary to CT and MRI. Ultrasound's ascent followed the growth in demand for real-time imaging, such as among researchers in developmental biology, cardiology, and solid tumor biology, who rely on ultrasound's high frame rate and Doppler mode to quantify the magnitude and direction of blood flow (4, 10, 11). The interest in preclinical ultrasound has been further catalyzed by new features that allow for 2D planar sonographs to be stacked into 3D images. This permits volume reconstruction previously achievable only with CT or MRI, but with image resolution that meets or exceeds that of micro-CT, plus superb soft tissue discrimination. In ultrasound imaging, sound waves emitted by a transmitter propagate through tissue before reflecting back to the transmitter. Sound waves ranging from 3 to 50 MHz produce images that reflect their variable transmission and reflection through different tissue types (4). Higher frequency probes resolve ever-finer details, and resolutions as low as 30 μm are possible with microbubble contrast agent (12).

The precision and accuracy of micro-CT and micro-ultrasound in 3D volume determination has been established by this and other laboratories, in applications ranging from fat graft volume measurement to tumor response in anti-cancer therapy (3, 11, 13-18). However, given their respective strengths in 3D reconstruction, there is no study to our knowledge that directly compares micro-CT to micro-ultrasound in volumetric analysis. In this study, we compare the performance of micro-CT to micro-ultrasound in the volumetric analysis of adipose tissue grafts in a murine model. We hypothesize that ultrasound is not inferior to micro-CT in this capacity, and that both modalities provide unique capabilities that confer different advantages depending on the experimental scenario.

Methods

Fat Harvesting / Grafting

In accordance with the Stanford University Institutional Review Board Guidelines, human lipoaspirate was obtained from a single female donor (age 62, BMI 28) with no significant medical comorbidities undergoing suction assisted lipectomy. Immediately upon receipt from the operating room, the lipoaspirate was twice washed with phosphate-buffered saline (Thermo Fisher Scientific; Waltham, MA) and centrifuged at 350 x g for five minutes, thus permitting separation and removal of excess blood and oil. The adipose tissue was kept refrigerated at 4° C until ready for grafting, and all grafting was performed on the same day as lipoaspirate harvest. Fifteen immunodeficient Crl:CD1-Foxn1nu CD-1® Nude mice (Charles River Laboratories, Inc; Wilmington, MA) were anesthetized with 2.5% isoflurane gas delivered through a mask. Two hundred microliter (200 µL) adipose tissue grafts were placed subcutaneously into the scalps of the CD-1 mice. All injections were performed using a 14-gauge cannula, and delivered in retrograde fashion as previously described by Chung *et al.* (17).

In vivo volumetric assessment

In vivo volumetric analysis was conducted at discrete time points (postoperative day 2, 15, 33, 42, 80 and 105), first by micro-CT and followed immediately by micro-ultrasound. The mice were anesthetized with 2.5% isoflurane gas delivered through a mask. After placing one mouse in each position of a four-basket harness, the mice were loaded into a Siemens Inveon PET / CT Multimodality System (Siemens AG; Munich DE) and scanned using a five-minute, 100 µm voxel resolution protocol. Three-dimensional reconstruction was accomplished using the Inveon Research Workplace (IRW) software suite (Siemens AG; Munich DE). A region of interest (ROI) was manually drawn around each fat graft on representative 2D sections; the set of ROIs for a given subject were combined to create a 3D reconstruction (Figure 1A). The software calculated volume and other detailed metrics on the 3D ROI.

Three-dimensional micro-ultrasound images were captured with a 40 MHz ultrasound transducer and the FujiFilm VisualSonics Vevo 2100 high-frequency digital imaging platform (FujiFilm VisualSonics Inc.; Toronto, Ontario, Canada). The mice were anesthetized with 2.5% isoflurane gas delivered through a mask. Cardiac status was monitored via stage-integrated electrocardiogram pads. Image capture was gated to

respiratory cycle to minimize motion artifact. Micro-ultrasound volume reconstruction and quantification was later accomplished on the Vevo LAB software suite. As in micro-CT, adipose tissue grafts were identified first on 2D projections, manually selected, and then digitally combined through software to produce the final 3D reconstruction (Figure 1B). The Vevo LAB software calculated volume.

Ex vivo volumetric assessment

Three mice were sacrificed at every time point except for the first (postoperative day 2). Grafts were explanted in their entirety through a dorsal incision made into the overlying scalp. Microdissection was performed to remove surrounding non-graft tissue. All explants were imaged via micro-CT and micro-ultrasound, as described above.

Statistics

Statistical analysis was performed on Prism (GraphPad Software, Inc.; La Jolla, CA). Intergroup variance was evaluated using Student's t-test. A *p*-value of less than 0.05 was considered statistically significant.

Results

All grafts were placed successfully and with no subsequent evidence of infection or rejection. Qualitative evidence of the differences in imaging between micro-CT and micro-ultrasound can be appreciated in Figure 2. Although both modalities yielded clear, high-resolution images, the improved resolution and contrast of ultrasound permitted unambiguous discrimination of graft from skin and calvarium, while also revealing the heterogeneous mix of soft tissue and oil within the graft itself (Figure 2B).

Mean volumes were calculated by averaging the volumes of all surviving mice at each experimental time point. The *in vivo* mean volumes over time determined by both micro-CT and micro-ultrasound are summarized in Figure 3. Although the volume measured by ultrasound is greater than that of CT at every time point, the difference never achieved statistical significance. Furthermore, t-test analysis revealed that when all *in vivo* volumes calculated at each time point are taken into consideration, the respective data sets created by micro-CT and micro-ultrasound are not significantly different ($p = 0.3569$).

Ex vivo mean volume over time was measured by micro-CT and micro-ultrasound, starting with the first graft explants on POD 15 (Figure 4). As in the *in vivo* data, ultrasound volume exceeded CT volume at every point, although not significantly. When t-test analysis was again applied to evaluate the cumulative volume measurements at all times, no significant difference was noted between CT and ultrasound ($p = 0.3856$).

The ratio of *in vivo* volume to explanted volume was calculated for every time point beginning with the first animal sacrifice on POD 15 (Figure 5). A ratio of 1.0 denotes equivalent measure of *in vivo* to *ex vivo* volume, and both micro-CT and micro-ultrasound *in vivo* to *ex vivo* volume ratios were not significantly different from 1.0 at all time points. Similar to the isolated *in vivo* and *ex vivo* analyses, the ratios determined by micro-CT at

each time point also did not differ significantly from the corresponding micro-ultrasound ratio ($p = 0.6883$).

Discussion

In our study, no significant difference between micro-CT and micro-ultrasound was appreciated when assessing volume of adipose tissue grafts in a murine model. In order to evaluate the accuracy of both modalities, mice were sacrificed at each time point, and the explanted volume – as quantified by CT or ultrasound – was compared to the corresponding *in vivo* volume. The fact that this ratio approaches 1.0 for both modalities and at all time points suggests near-equivalency in measured volume, *in vivo* and *ex vivo*. Moreover, if we regard the explanted volume as the graft's true volume, the ratio of *in vivo* to *ex vivo* volume across all animals and time points illustrates parity in accuracy between CT and ultrasound.

These results would not necessarily be predicted based on the documented capabilities of both machines: the micro-CT scanner captured images at 100- μm resolution while the 40 MHz micro-ultrasound probe imaged at 40- μm resolution. The ability of this particular micro-CT protocol to compete on par with ultra-high frequency ultrasound is in part attributable to the study design: a single, experienced micro-CT and micro-ultrasound operator performed all imaging acquisition, interpretation, and ROI definitions. This eliminated inter-operator variability, and allowed for subtle discrimination of the target adipose tissue from the nearly isodense periphery in the considerably lower contrast CT scans. The ultrasound images, in comparison, were of greatly improved sensitivity, contrast and resolution. This made much easier the task of precisely defining the region of the adipose tissue graft. As previously alluded to, the increased resolution did come at some cost, as the range of spatial resolution was more limited in depth compared to the lower frequency, reduced resolution probes. Still, the roughly 3-cm penetration was adequate for the more superficial grafting performed in our murine scalp model.

Sensitivity of and soft tissue discrimination in the micro-CT images can be improved to the level of micro-ultrasound through the use of contrast, although this may not necessarily illuminate the architecture of the structures within early avascular fat tissue grafts used in this experiment. In this regard, micro-ultrasound displayed one of its greatest strengths. The fidelity of ultrasonography allowed for better appreciation of the inherent heterogeneity of the graft itself. Whereas these details were commonly attenuated in the relative isodensity of the micro-CT images, subtle changes in tissue texture, density, and distribution were evident in the ultrasound images. Moreover, the interface between solid tissue and liquid from oil, water, or fat necrosis, was more readily observed in micro-ultrasound. While the risk for an immunogenic response was reduced in this study through the use of immunodeficient mice, in other animal models where xenogenic fat grafting may induce more inflammation, this capability of ultrasound would be particularly advantageous to visualize local fluid mobilization and intra-graft cyst development.

Where micro-CT lacked in soft tissue discrimination, it excelled in speed and ease of use. The imaging sled of the CT scanner accepted a multi-mouse adapter, which enabled us to image four mice at a time using a five-minute scanning protocol. Imaging the animals with

the micro-ultrasound took considerably more time and expertise. Only one mouse could be imaged by the ultrasound at any given time, and probe configuration had to be customized and fine-tuned for each subject to ensure proper travel over the entire graft. This may introduce variability based on the equipment operator, as ultrasound acquisition must be configured to each mouse individually. Once properly configured, however, imaging with micro-ultrasound was assisted by computer controlled automated acquisition.

Importantly, micro-ultrasound does avoid radiation exposure to experimental animals, a consideration which becomes important with serial imaging in longitudinal studies. A major drawback to micro-CT, significant ionizing radiation can be imparted on subjects. In higher-resolution CT scans, radiation exposure approaches 0.5 Gy, a tenth of the dose recognized as lethal to 50% of mice 30 days after exposure (1), and worsens with cumulative exposure over multiple scans. Additionally, ionizing radiation can unexpectedly alter the immune system of experimental subjects which may impact retention of xenotransplanted fat grafts (1, 5).

Finally, although it was not evaluated in this study, micro-MRI can provide improved soft tissue visualization compared to micro-CT and micro-ultrasound. However, CT and ultrasound offer much faster scanning times and considerable economy over MRI (1, 3). For these reasons, micro-CT and micro-ultrasound are both better suited for high-volume, multi-animal experiments.

Conclusion

Micro-ultrasound and micro-CT yield similar three-dimensional volumetric analyses. Micro-ultrasound is portable, inexpensive, and produces images of the highest resolution and contrast if high-frequency probes are employed. Nevertheless, 3D ultrasound set-up can be time-consuming and the automated acquisition does not necessarily eliminate inter-operator variability. Micro-CT is fast, economical, and is capable of resolutions comparable to micro-ultrasound. Still, non-contrast CT is plagued by poor soft tissue discrimination, and the ionizing radiation delivered causes tissue damage and may potentially alter the subject's immunobiology. Thus, in evaluating the use of either micro-CT or micro-ultrasound, the choice should be based on the requirements of the experimental design, not simply the inherent benefits and disadvantages of each modality. Ultimately, from the perspective of 3D volume reconstruction and analysis, both micro-CT and micro-ultrasound will be accurate and valuable adjuncts to any comparative investigation.

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Figure 1. Three-dimensional reconstruction and volumetric analysis of micro-CT images using Inveon Research Workplace (**A**) and micro-ultrasound images with Vevo LAB (**B**).

Figure 2. Soft tissue discrimination in micro-CT versus micro-ultrasound. Note the relative isodensity of the micro-CT image (**A**). In comparison, the improved sensitivity and contrast of micro-ultrasound uncovers the inherent heterogeneity of the graft (**B**).

Figure 3.

In vivo mean volume over time. The differences between micro-CT and micro-ultrasound at each time point were not statistically significant ($p = 0.3569$).

Figure 4.

Ex vivo mean volume over time. The differences between micro-CT and micro-ultrasound were not statistically significant ($p = 0.3856$).

Figure 5. Ratio of *in vivo* volume to the corresponding explanted volume. The differences between micro-CT and micro-ultrasound were not statistically significant ($p = 0.6883$). A ratio of 1.0 denotes equivalent measure of *in vivo* to *ex vivo* volume.